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Letter to the Editor

The Asp298 but not the C-786 genotype of the endothelial nitric oxide synthase is reduced with age in healthy Swiss men

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NO is produced by a family of enzymes called the NO synthases which includes the endothelial NO synthase (eNOS), the neuronal NO synthase (nNOS) and the inducible NO synthase (iNOS).

In endothelial cells, eNOS is constitutively expressed and the physiological role of endothelial-derived NO seems to be the control of vascular tone and the preservation of endothelial function (1). Moreover, NO causes suppressed platelet aggregation and decreased monocyte and lymphocyte adhesion to endothelium, suggesting that endothelium-derived NO has antithrombotic activities and decreases infiltration of monocytes and lymphocytes into the intima of arteries (2). In addition, NO reduces smooth muscle cell migration and proliferation. All these regulatory functions of NO counteract atherogenesis and suggest an anti-atherosclerotic effect of NO (3).

Several polymorphisms were detected in the *eNOS* gene including two which may result in reduced eNOS activity. The T-786C polymorphism in the *eNOS* promoter region was shown to result in reduced transcriptional activity of the minor *C-786* allele (4). The Asp298 eNOS protein of the Glu298Asp polymorphism in exon 7 showed a lower protein activity in human placentas than the Glu298 eNOS protein and preferential degradation in vitro (5, 6). However, other recent in vitro studies questioned the functionality of the Asp298 mutation in eNOS because there was no difference in expression, protein activity or cellular localization between the two eNOS variants (7, 8).

A recent meta-analysis suggests that there is an association of the homozygous Asp298 *eNOS* genotype with atherosclerosis, but not of the homozygous C-786 polymorphism (9). We decided to use a different approach to investigate the association of the homozygous C-786 and the homozygous Asp298 *eNOS* genotypes with age-related disease. We surmised that the frequency of any deleterious polymorphic allele will decrease in the older population if this allele accelerates late onset disease but does not affect reproductivity. In contrast, polymorphisms at genetic loci that carry no selective disadvantage should exhibit frequencies that remain constant throughout life (10). If the C-786 or the Asp298 *eNOS* genotype were significant risk factors for age-related disease, then one would expect that the frequency of the homozygous genotype of these alleles decreases with age in the healthy population. Therefore, we compared the genotype distribution of the two polymorphisms between younger (20–54 years) and older (55–70 years) healthy volunteers in the Swiss population.

A total of 400 healthy blood donors (aged 19–70 years) who gave informed consent to participate in the study were randomly selected. Exclusion criteria of the Swiss Red Cross were applied including coronary artery disease (CAD), stroke, diabetes, claudication, untreated hypertension, anemia, infection, cancer, chronic kidney disease, neurological disease and others. Plasma creatinine (91.6 ± 11.1 μmol/L for males and 79.6 ± 9.3 μmol/L for females) and total cholesterol levels (5.6 ± 1.1 mmol/L) were in the range of the healthy Swiss population. The genotypes for the T-786C and Glu298Asp *eNOS* polymorphisms were analyzed by tetra-primer PCR assays (11).

The allele frequencies for the *eNOS* T-786C polymorphism were 57% (T-786) and 43% (C-786) and the frequencies for the Glu298Asp polymorphism were 65.4% (Glu) and 34.6% (Asp). For both polymorphisms the genotype frequencies were in agreement with those predicted by the Hardy-Weinberg equilibrium and are comparable to those in other Europeans (12).

When we analyzed the Glu298Asp genotype according to age and gender, using a recessive model (Table 1), the homozygous Asp298 genotype was significantly less frequent in males ≥ 55 years (oldest tercile) than in males < 55 years (5.95% vs. 16.35%, $p = 0.015$), whereas no notable difference was seen between women older and younger than 55 years (11.76% vs. 11.32%, $p = 0.935$). To test whether this difference is sensitive to the selected age cut-off, we performed a secondary analysis of the data with an age cut-off set to 60 years (oldest quintile). The frequency of the homozygous Asp298 genotype further

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Table 1 Genotype frequencies for the Glu298Asp and the T-786C polymorphisms in *eNOS* according to age and gender.

Gender	Age, years	GluGlu and GluAsp	AspAsp	TT and TC	CC
Male	<55 (n=159)	133/159	26/159	131/159	28/159
		83.65%	16.35%*	82.39%	17.61%
	≥55 (n=84)	79/84	5/84	72/84	12/84
		94.05%	5.95%*	85.71%	14.24%
Female	<55 (n=106)	94/106	12/106	87/106	19/106
		88.68%	11.32%	82.08%	17.92%
	≥55 (n=51)	45/51	6/51	47/51	7/51
		88.24%	11.76%	86.27%	13.73%

*Significant difference compared to GluGlu and GluAsp ($p=0.015$).

decreased to 5.08% in men ≥60 years (5.08% vs. 15.22%, $p=0.027$) but remained unchanged in women ≥60 years (10.53% vs. 11.76%, respectively, $p=0.833$). The genotype frequencies in the different subgroups were all in agreement with those predicted by the Hardy-Weinberg equilibrium.

Analysis of T-786C polymorphisms in *eNOS* according to age and gender showed no significant difference in genotype frequency between the groups. The CC genotype was slightly reduced at higher age (≥55 years) in males and females (Table 1) but was similar when the age cut-off was set to 60 years (data not shown).

This study shows that the homozygous Asp298 genotype in the *eNOS* gene is reduced in the older healthy male population. A population in Hardy-Weinberg equilibrium for a particular polymorphism should display genotype frequencies that remain stable throughout life (10). Any decrease in genotype frequency with age in the healthy population is expected only if the genotype is associated with disease which develops past the age of reproduction. Atherosclerosis is such a disease, which in general does become clinically overt in the fifth to sixth decade of life and eNOS-dependent NO production is thought to be atheroprotective through its preservation of endothelial function.

The decrease in the homozygous Asp298 genotype frequency in older men was observed in blood donors who went through a selection process excluding volunteers with various disorders. Therefore, combined mortality and morbidity from various disorders reduced the Asp298 genotype in this selected healthy population. However, considering that eNOS is predominantly expressed in the vasculature and the heart (13), it is most likely that mortality and morbidity from cardiovascular disease are the underlying cause for the reduction of the homozygous Asp298 *eNOS* genotype in older men. This raises the question whether mortality and morbidity from atherosclerotic disease can account for the observed reduction in homozygous Asp298 genotypes in older males. In Switzerland, the cumulative mortality from CAD in men aged 20–54 years and 55–70 years is 0.17% and 1.0%, respectively, and in women the cumulative mortality from CAD is 0.003% and 0.25%, respectively. Morbidity data for atherosclerosis are not available in Switzerland. However, we can estimate the prevalence for atherosclerosis. In men aged 20–54 years and 55–70 years, two independent estimates yielded a prevalence for atherosclerosis of 1.3–3.8% and 12.8–15.6%, respectively, and in women of 0.4–0.5% and 4.5–6.3%, respectively. For both genders, the estimated prevalence in the older groups is slightly lower than the prevalence for CAD in the Framingham study in the age group 55–64 years (14). Therefore, it appears plausible that mortality and morbidity from atherosclerotic disease may account for the significant reduction of homozygous Asp298 genotypes in older healthy males.

While we observed a decrease in homozygous Asp298 genotypes in males, there was no difference in genotype frequencies among women. Considering that the average age in our male and female group was similar, this may be explained by the low morbidity rate for CAD in premenopausal women and the approximate 10-year difference in mortality from CAD between the sexes (15). Both of our estimates of prevalence for atherosclerosis in women also reflect this difference between the sexes.

The study has several potential limitations. Because this study is cross-sectional it is not possible to assess the cause of the decrease in the Asp298 genotype with advancing age. Since cardiovascular disease is the leading cause of morbidity and death in subjects over 60 years of age, and eNOS is implicated in vascular function and integrity, the most likely cause of the observed decrease in the homozygous Asp298 genotype with advancing age are cardiovascular diseases. However, we cannot exclude that other diseases associated with the homozygous Asp298 genotype also contribute to the decreased prevalence of this genotype in older men. An additional limitation is the limited power of our study. For example, a 50% decrease in prevalence of the CC genotype in the older age group is required for the T-786C polymorphism to detect a difference with a power of 80% and smaller decreases will be missed more frequently. Therefore, we cannot exclude smaller real age-related decreases in the genotype frequency with sufficient power.

In summary, our study in healthy volunteers shows that the homozygous Asp298 *eNOS* genotype is diminished in the older male population, whereas no difference was found in allele frequency between the younger and the older for the T-786C polymorphism. These findings show that homozygous Asp298 *eNOS* genotype is associated with disease that develops past the age of reproduction.

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